CHAPTER FIVE

5. SCREENING FOR ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY BY AGAR DIFFUSION METHOD

5.1 Introduction

There is an increasing need for safe and effective antibacterial and antifungal agents to fight the continuously emerging resistant pathogenic microbes. This need is particularly great in immunocompromised patients who are at risk to infection by the opportunistic pathogens such as *Staphylococci* and *Candida* (Gordee *et al.*, 1984).

Historically most antibacterial and antifungal agents have been discovered by screening soil, sewage, and other products from naturally occurring antibiotic-producing microorganisms. Fruiting bodies of the fungus *G. lucidum* and its related species *G. tsugae* have been used as crude drug in traditional medicine for treatment of various ailments in China, Japan and other Asian countries (Mizuno *et al.*, 1995). This is because the crude drug has been known to contain a very rich source of bioactive compounds (Jong and Birmingham, 1992).

In this study, the aim was to screen the fractions collected from the column chromatography for antibacterial and antifungal properties. The significance of the test microorganisms used is summarized in Table 5.1. The bioassay was conducted using the classical paper disc diffusion method. If performed with adequate controls, this method is as accurate as more complicated test available today (Bridson, 1995).

Table 5.1: Significance of the test microorganisms.

Organism tested	Nature	Morphology and Gram reaction	Important infections caused by individual organism/ comment
Bacillus cereus, Bacillus subtilis	Aerobic	Bacilli (rod-shape), Gram-positive	Conjunctivitis
Staphylococcus aureus	Aerobic	Cocci (round-shape), Gram-positive	Serious suppurative infections and systemic disease, toxins produced can cause food poisoning and toxic shock
Pseudomonas aeruginosa	Aerobic	Bacilli, Gram-negative	Wound infection, urinary tract infection
Escherichia coli	Aerobic	Bacilli, Gram-negative	Various infections including wound, urinary tract infection and epidemic diarrhea (especially in children)
Candida albicans	Facultative	Yeast-like fungus	Commensal of mouth, causes superficial mycoses
Schizosaccharomyces pombe	Facultative	Fission yeast	Harmless, rapidly growing eukaryote that is popular model system to understand basic biological processes. The bioassay tested enable us to have some insight on eukaryotic system (e.g antitumour activity)

Source: compiled from Davis et al. (1980)

5.2 Materials and Methods

5.2.1 Test bacteria.

Bacillus cereus, Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa and *Escherichia coli* were used as test bacteria in this study. All the

bacteria were obtained from Institute of Postgraduate Studies and Research, University Malaya. These bacterial strains were maintained on Nutrient Agar (NA, Difco) plate.

5.2.2 Test fungi.

Candida albicans and *Schizosaccharomyces pombe* were used as the test fungi. *Candida albicans* was obtained from Institute of Medical Research, Malaysia and *S. pombe* was obtained from Institute of Biological Science, University Malaya. *Candida albicans* and *S. pombe* were maintained on Saboraud Dextrose agar (SDA, Difco) and Yeast Peptone Glucose agar (YPG) (Appendix A) respectively.

5.2.3 Screening for antibacterial and antifungal activity

The classical paper disc diffusion method was used as the screening method (Nadaraj, 1996). Whatman paper discs of 6 mm diameter impregnated with 12 µl of a specific fraction was then placed onto the agar surface. The bioassay was performed in triplicate. Both negative (the solvent only) and positive (chloramphenicol 50 µg/ml for bacteria and nystatin 50 µg/ml for fungi) controls were included in each test plate. The test plates were incubated at $37^{\circ} \pm 2^{\circ}C$ for all the test bacteria and for *C. albicans* but at $27^{\circ} \pm 2^{\circ}C$ for *S. pombe*. The diameter of zone of inhibition of growth, if any, for each test plate was recorded after 24 h of incubation.

5.3 Results and Discussion

5.3.1 Activity of overall fractions

In this study, the fractions collected from column chromatography of hexane and methanol crude extracts were screened for antibacterial and antifungal activity. The results (Table 5.2 and 5.3) showed that no activity was found in hexane crude extract against all the test microorganisms, while the methanol crude extract showed weak (7.0-8.9 mm) to moderate (9.0-10.9 mm) activity against all the test microorganisms.

Further investigations showed that all the fractions collected after column chromatography of hexane and methanol crude exhibited some activity. Of the 52 fractions tested, 25 showed moderate activity (9.0-10.9 mm), while the other fractions showed weak activity (7.0-8.9 mm) against one or more of the test microorganisms. None of the fractions exhibited good activity (>11.0 mm) comparable to the standard antibiotic, chloramphenicol (50 μg/ml) or antifungal agent, nystatin (50 μg/ml), which was approximately 19.0-20.0 mm (Table 5.2 and 5.3).

Table 5.2: Antibacterial activity of the fractions from *G. tsugae*

Sample	*Diameter of zone of clearance (mm)				
	B. cerues	*B. subtilis*	*P.aeru ginosa*	*S. aureus*	*E. coli*
crude (Hex)	6.67 ±0.58	6.67 ±0.58	6.50 ±0.50	6.67 ±0.58	6.00 ±0.00
crude (MeOH)	7.33 ±0.58	9.00 ±0.00	7.00 ±0.00	8.00 ±1.00	9.00 ±0.00
Hex Fr					
Fr1-2	7.00 ±0.00	6.33 ±0.58	6.33 ±0.58	6.33 ±0.58	6.00 ±0.00
GM 1(Fr 3-6)	6.33 ±0.58	6.00 ±0.00	6.50 ±0.50	6.00 ±0.00	7.33 ±0.58
Fr 7-9	7.67 ±1.15	6.00 ±0.00	6.00 ±0.00	6.00 ±0.00	6.00 ±0.00
GM 2 (Fr 10-11)	6.00 ±0.00	7.00 ±0.00	7.00 ±0.00	7.33 ±0.58	7.67 ±0.58
GM 3 (Fr 12-13)	6.00 ±0.00	6.67 ±0.58	6.50 ±0.50	6.67 ±0.58	6.33 ±0.58
GM 4 (Fr 14-15)	9.00 ±1.00	6.00 ±0.00	6.33 ±0.58	7.33 ±0.58	6.67 ±1.15
GM 5 (Fr16)	8.33 ±0.58	6.33 ±0.58	6.67 ±0.58	6.67 ±0.58	7.00 ±1.00
Fr 17	9.00 ±0.00	9.33 ±0.58	6.00 ±0.00	8.00 ±0.00	8.33 ±1.15
Fr 18	8.33 ±0.58	7.00 ±0.00	6.00 ±0.00	7.33 ±0.58	7.00 ±0.00
Fr 19	8.33 ±0.58	8.00 ±0.00	7.00 ±0.00	7.33 ±0.58	8.33 ±0.58
GM 1.1(Comp1)	8.00 ±0.58	8.00 ±0.00	6.00 ±0.00	6.00 ±0.00	6.67 ±0.58
GM 1.2-1.3	6.00 ±0.00	6.00 ±0.00	6.67 ±0.58	6.00 ±0.00	6.67 ±0.58
GM 1.4	7.67 ±0.58	7.00 ±0.00	6.67 ±0.58	6.67 ±0.58	6.67 ±0.58
GM 1.5	6.33 ±0.58	7.00 ±0.00	6.50 ±0.00	6.00 ±0.00	6.67 ±0.58
GM 1.6	7.67 ±0.58	7.00 ±0.00	7.00 ±0.00	7.00 ±0.00	6.67 ±0.58
GM 2.2(DEHP)	9.00 ±1.00	9.67 ±0.58	8.00 ±0.00	8.33 ±0.58	7.67 ±1.15
GM 2.4	6.67 ±0.58	7.00 ±0.00	6.00 ±0.00	7.00 ±1.00	7.00 ±0.00
GM 2.5	7.50 ±0.50	6.33 ±0.58	6.33 ±0.58	7.00 ±1.00	7.67 ±0.58
GM 2.6	6.00 ±0.00	8.67 ±0.58	7.33 ±0.58	7.00 ±0.00	7.50 ±0.87
GM 3.1-3.5	9.67 ±0.58	8.33 ±1.15	9.17 ±1.04	10.17 ±0.76	8.33 ±0.58
GM 3.6-3.11	8.00 ±1.00	8.67 ±0.58	7.00 ±0.00	8.33 ±1.15	8.67 ±1.53
GM 3.12-3.18	7.00 ±0.00	7.00 ±1.00	6.00 ±0.00	7.67 ±1.15	8.17 ±0.29
GM 3.19	9.50 ±0.50	7.67 ±0.58	7.67 ±0.58	9.00 ±1.73	9.83 ±0.29
GM 3.1.1	7.33 ±1.15	7.00 ±1.00	7.33 ±0.58	6.67 ±1.15	8.17 ±1.26
GM 3.1.2(DEHP)	8.33 ±0.58	9.50 ±0.50	8.00 ±0.00	8.67 ±0.58	8.00 ±1.00
GM 3.1.3	6.33 ±0.58	7.67 ±1.53	6.67 ±0.58	6.67 ±1.15	7.33 ±0.58
GM 3.1.4	7.00 ±1.00	7.00 ±1.00	7.00 ±0.00	6.00 ±0.00	7.00 ±1.00
GM 3.1.5	6.00 ±0.00	7.00 ±0.00	6.33 ±0.58	6.00 ±0.00	7.00 ±0.00
GM 3.1.6	8.00 ±0.00	7.00 ±0.00	7.00 ±0.00	10.33 ±0.76	7.00 ±0.00
GM 3.6.1	7.33 ±0.58	7.67 ±0.58	6.33 ±0.58	8.67 ±1.53	7.00 ±0.00
GM 3.6.2	9.00 ±0.00	6.33 ±0.58	6.00 ±0.00	6.33 ±0.58	6.00 ±0.00
GM 3.6.3	8.00 ±1.00	7.67 ±0.58	7.67 ±0.58	8.33 ±1.53	7.67 ±1.15
GM 3.6.4	8.50 ±0.87	8.33 ±1.15	8.67 ±0.58	8.33 ±1.15	7.33 ±0.58
GM 3.6.5 (stellasterol)	6.00 ±0.00	7.00 ±0.00	6.17 ±0.29	6.67 ±0.58	6.00 ±0.00
GM 3.6.6	7.00 ±0.00	7.00 ±0.00	7.00 ±1.00	6.00 ±0.00	7.00 ±0.00

Continue from Table 5.2

Sample	Diameter of zone of clearance (mm)				
	B. cerues	*B. subtilis*	*P.aeru ginosa*	*S. aureus*	*E. coli*
GM 4.1	8.33 ±1.15	7.33 ±0.58	6.67 ±0.58	8.67 ±0.58	7.67 ±0.58
GM 4.2	8.67 ±0.58	8.33 ±0.58	6.67 ±0.58	8.33 ±0.58	7.00 ±1.00
GM 4.3 (ergosterol)	6.33 ±0.58	6.67 ±0.58	6.00 ±0.00	7.00 ±0.00	7.00 ±0.00
GM 4.4	8.50 ±0.87	9.00 ±1.00	8.00 ±0.00	8.33 ±0.58	8.00 ±1.00
GM 5.1	9.33 ±1.15	8.00 ±1.00	9.00 ±1.00	8.33 ±0.58	8.00 ±0.00
GM 5.2	7.00 ±0.00	7.33 ±0.58	9.67 ±0.29	7.00 ±0.00	7.33 ±0.58
GM 5.3	7.67 ±0.58	7.33 ±0.58	7.00 ±1.00	8.33 ±0.58	7.00 ±0.00
GM 5.4	6.67 ±0.58	6.00 ±0.00	7.00 ±0.00	8.67 ±0.58	6.67 ±0.58
MeOH Fr					
Fr 1	8.33 ±1.15	8.67 ±0.58	8.67 ±0.58	8.67 ±1.15	8.17 ±1.26
Fr 2	7.00 ±0.00	9.00 ±1.00	8.33 ±0.58	7.33 ±0.58	7.83 ±0.76
Fr 3	7.67 ±0.58	8.33 ±0.58	8.00 ±0.00	9.00 ±1.00	7.67 ±0.58
Fr 4	7.33 ±1.53	7.00 ±1.00	8.33 ±0.58	7.83 ±0.76	7.67 ±0.58
Fr 5	8.00 ±0.00	8.83 ±0.76	10.67 ±0.58	9.33 ±1.15	6.00 ±0.00
Fr 6	7.67 ±0.58	8.67 ±0.58	9.5 ±0.50	8.67 ±0.58	7.67 ±0.58
Fr 7	6.00 ±0.00	6.00 ±0.00	9.5 ±0.50	8.83 ±0.29	7.67 ±0.58
Fr 8	9.00 ±0.00	8.67 ±0.58	9.33 ±0.58	10.00 ±0.00	8.00 ±1.00
Fr 9	7.00 ±0.00	8.33 ±0.58	6.33 ±0.58	7.33 ±0.58	8.00 ±0.00

* Diameter 7.0 to 8.9mm = weak activity, 9.0 to 10.9 = moderate activity and >11.0 mm = good activity. Standard antibiotic, chloramphenicol (50 ug/ml) = 19.0 to 21.0 mm. Raw data in Appendix II

Table 5.3: Antifungal activity of the fractions from *G. tsugae*

Sample	Diameter of zone of clearance (mm)	
	C. albicans	*S. pombe*
crude (Hex)	6.00 ±0.00	6.00 ±0.00
crude (MeOH)	7.00 ±0.00	7.00 ±0.00
Hex Fr		
Fr1-2	6.67 ±0.58	6.00 ±0.00
GM 1(Fr 3-6)	6.67 ±0.58	7.00 ±0.00
Fr 7-9	6.00 ±0.00	6.00 ±0.00
GM 2 (Fr 10-11)	7.33 ±0.58	6.67 ±0.58
GM 3 (Fr 12-13)	6.33 ±0.58	6.00 ±0.00
GM 4 (Fr 14-15)	7.67 ±0.58	8.00 ±1.00
GM 5 (Fr16)	6.67 ±0.58	6.00 ±0.00
Fr 17	7.00 ±0.00	6.33 ±0.58
Fr 18	6.00 ±0.00	6.33 ±0.58
Fr 19	8.00 ±0.00	7.33 ±0.58
GM 1.1(Compd1)	6.67 ±0.58	7.00 ±1.00
GM 1.2-1.3	6.83 ±0.29	7.00 ±1.00
GM 1.4	6.67 ±0.58	8.17 ±0.29
GM 1.5	6.67 ±0.58	7.67 ±0.58
GM 1.6	6.00 ±0.00	7.00 ±0.00
GM 2.2(DEHP)	9.83 ±0.29	9.00 ±1.00
GM 2.4	7.33 ±0.58	6.67 ±0.58
GM 2.5	6.67 ±0.58	7.00 ±0.00
GM 2.6	6.67 ±0.58	6.67 ±0.58
GM 3.1-3.5	9.00 ±0.00	9.33 ±0.58
GM 3.6-3.11	9.50 ±0.50	8.33 ±1.15
GM 3.12-3.18	7.00 ±1.00	6.00 ±0.00
GM 3.19	8.00 ±0.00	7.00 ±0.00
GM 3.1.1	9.50 ±0.50	9.00 ±1.00
GM 3.1.2(DEHP)	9.83 ±0.29	10.30 ±1.53
GM 3.1.3	9.00 ±1.00	7.67 ±1.15
GM 3.1.4	7.00 ±0.00	8.33 ±1.15
GM 3.1.5	7.00 ±0.00	6.00 ±0.00
GM 3.1.6	9.33 ±0.58	9.17 ±0.29
GM 3.6.1	7.33 ±0.58	7.33 ±0.58
GM 3.6.2	9.00 ±1.00	6.67 ±0.58
GM 3.6.3	9.00 ±0.00	8.33 ±0.58
GM 3.6.4	8.50 ±0.00	8.33 ±1.15
GM 3.6.5	6.33 ±0.58	6.67 ±0.58
GM 3.6.6	6.67 ±0.58	8.00 ±0.00
GM 3.6.5(stellasterol	8.33 ±0.58	7.67 ±0.58
GM 3.6.6	6.67 ±0.58	8.00 ±0.00

Continue from Table 5.3

Sample	Diameter of zone of clearance (mm)	
	C. albicans	*S. Pombe*
GM 4.1	10.00 ±0.50	8.83 ±0.76
GM 4.2	10.00 ±1.00	8.00 ±1.00
GM 4.3 (ergosterol)	6.33 ±0.58	6.67 ±0.58
GM 4.4	9.67 ±0.58	9.50 ±0.50
GM 5.1	7.83 ±0.29	6.67 ±0.58
GM 5.2	8.17 ±0.29	7.33 ±0.58
GM 5.3	7.33 ±0.58	6.33 ±0.58
GM 5.4	8.50 ±0.50	6.50 ±0.50
MeOH Fr		
Fr 1	9.33 ±0.58	8.33 ±0.58
Fr 2	9.67 ±0.58	8.33 ±0.58
Fr 3	9.00 ±1.00	8.50 ±0.87
Fr 4	9.50 ±0.50	8.00 ±1.00
Fr 5	9.00 ±1.00	7.33 ±0.58
Fr 6	7.67 ±1.15	7.33 ±0.58
Fr 7	8.50 ±0.50	8.00 ±0.00
Fr 8	9.33 ±0.58	7.33 ±0.58
Fr 9	6.67 ±0.58	7.33 ±0.58

* Diameter 7.0 to 8.9mm = weak activity, 9.0 to 10.9 = moderate activity and >11.0 mm = good activity. Standard antifungal agent, nystatin (50 ug/ml) = 19.0 to 20.0 mm. Raw data in Appendix II

Candida albicans was found to be the most susceptible test microorganism, with 18 fractions showing moderate activity against this opportunistic pathogen. In contrast, *E. coli* was observed to be the least susceptible test microorganism, with only 2 fractions showing moderate activity against this Gram-negative bacterium (Table 5.4).

A total of six fractions showed moderate inhibition against *B. cereus*, *B. subtilis*, *S. aureus* and *S. pombe*, while a total of eight fractions showed moderate inhibition against *P. aeruginosa* (Table 5.4). Based on these results, the fractions were further divided into groups (Table 5.5). Fractions GM 3.1-3.5, Fr 5 (MeOH) and Fr 8 (MeOH) were found to exhibit broad-spectrum activity against both Gram-positive and Gram-negative bacteria as well as the test fungi were grouped as Group III (Table 5.5).

In general, the methanol fractions and the more polar fractions showed an overall greater activity when compared to the hexane fractions and non-polar fractions (Table 5.2 and 5.3). This may be due to the fact that many of the reported biological active ganoderic acids and lucidenic acids described earlier are mostly present in the more polar methanol extract (Jong and Birmingham, 1992).

The activity of the crude extract should be the sum of the compounds contained in the fractions (Farnsworth and Bingel, 1977). However, the results showed that the hexane crude gave no activity as compared to its fractions.

Table 5.4: Fractions that show moderate inhibition (i.e. inhibition zones of 9 mm and above) to one or more of the test organism.

Sample	*B.c	B.s	S.a	P.a	E.c	C.a	S.p
Hexane Fr							
Fr 17 **(Hex)	+	+	-	-	-	-	-
GM 2.2	-	+	-	-	-	+	+
GM3.1-3.5	+	-	+	+	-	+	+
GM3.6-3.11	-	-	-	-	-	+	-
GM3.19	+	-	+	-	+	-	-
GM3.1.1	-	-	-	-	-	+	+
GM3.1.2	-	+	-	-	-	+	+
GM3.1.3	-	-	-	-	-	+	-
GM3.1.6	-	-	+	-	-	+	+
GM3.6.2	+	-	-	-	-	+	-
GM3.6.3	-	-	-	-	-	+	-
GM4.1	-	-	-	-	-	+	-
GM4.2	-	-	-	-	-	+	-
GM4.4	-	+	-	-	-	+	+
GM5.1	+	-	-	+	-	-	-
GM5.2	-	-	-	+	-	-	-
MeOH crude(1)	-	+	-	-	+	-	-
Fr 1 (MeOH)	-	-	-	-	-	+	-
Fr 2 (MeOH)	-	+	-	-	-	+	-
Fr 3 (MeOH)	-	-	+	-	-	+	-
Fr 4 (MeOH)	-	-	-	-	-	+	-
Fr 5 (MeOH)	-	-	+	+	-	+	-
Fr 6 (MeOH)	-	-	-	+	-	-	-
Fr 7 (MeOH)	-	-	-	+	-	-	-
Fr 8 (MeOH)	+	-	+	+	-	+	-
Total inhibition	*6*	*6*	*6*	*8*	*2*	*18*	*6*

**B.c= B. cereus, B.s= B. subtilis, S.a= S. aureus, P.a= P. aeruginosa, E.c= E. coli, C.a= C. albicans S.p= S. pombe* **Hex= hexane fraction, MeOH= methanol fraction

Table 5.5: Grouping of the fractions that showed moderate inhibition

Group I*	Group II	Group III
GM 3.1-3.5	GM 3.1-3.5	GM 3.1-3.5
GM 3.19	GM 3.1.1	Fr 5 (MeOH Fr)
GM 5.1	GM 3.1.2/GM 2.2	Fr 8 (MeOH Fr)
MeOH crude	GM 3.1.6	
Fr 5 (MeOH Fr)	GM 4.4	
Fr 8 (MeOH Fr)		

*Group I=inhibit both Gram +ve & -ve bacteria
Group II=inhibit both fungi
Group III=inhibit both Gram +ve & -ve bacteria & fungus.

One possible explanation is that the active compound (s) was (were) present in low concentration in the crude extract to manifest activity at the dose levels employed. After separation by column chromatography, the concentration of the active compounds in the fractions will increase, exhibiting activity against the test microorganisms.

If the active compound (s) was (were) present at sufficient concentration in the hexane crude, another possible explanation is that there may be some component (s) present, which possess suppressive effect (antagonize) towards the active compound (s). The possibility of having antagonistic substances present in the crude extract is likely since it was prepared with a single solvent, that is, hexane.

To overcome such problem, the hexane crude should be further extracted with different solvents, such as chloroform and methanol. In this way, the extract is separated into medium polarity (chloroform-soluble) and then higher polarity (methanol soluble) (Farnsworth and Bingel, 1977).

The antagonistic effect was also clearly exemplified in GM 2. Before column chromatography, GM 2 showed very weak activity against the *Bacillus* spp. (Table 5.2). However, after column chromatography, the fraction GM 2.2 (DEHP) showed a marked increase in activity against *B. cereus* (9.00 mm) and *B. subtilis* (9.67 mm) as compared to GM 2, as well as to the other collected fractions, GM 2.4, 2.5 and 2.6 (Table 5.2).

On the other hand, synergistic effects were also being observed in the fractions. For example, GM 3.1-3.5 showed a broad-spectrum activity against all the test microorganisms. However, after column chromatography, the fractions collected, GM 3.1.1, 3.1.2, 3.1.3, 3.1.4, 3.1.5 and 3.1.6 showed a decrease in their respective activity against most of the test microorganisms (Table 5.2 and 5.3).

Both the antagonistic and synergistic effects play an important role in the activity of the fractions. As a result of these effects, different combinations of compounds in the fractions gave rise to different strengths of inhibition against the different types of test microorganisms (Table 5.2 and 5.3).

Chen et al. (1992) had reported *in vivo* inhibition of *C. albicans*, but did not identify the bioactive compounds responsible of the inhibition. From the result (Table 5.4), the 18 fractions that showed moderate activity against *C. albicans* were mostly terpenoids. Further purification of the 18 fractions will be promising since the possibility of obtaining at least an effective antifungal agent against *C. albicans* is very high.

Among the test microorganisms, *S. cerevisiae* is harmless. This yeast was chosen as test organism because many of the genes that regulate normal cell division in complex organisms such as humans also exist and function the same way in this yeast (Griffin, 1994). A number of yeast cell cycle and DNA repair genes have been found to be directly involved in human cancer when they malfunction (Davis *et al.*,

1980). Since the fission yeast cells can grow quickly, with generation time between 2-4 hours and are easy to manipulate in the laboratory, it can thus be used as a good source of primary screening for antitumor activity.

From Table 5.4, six fractions showed moderate activity against *S. pombe*. This result is in accordance to Jan and Kim (1987), who reported the extract of *G. lucidum* had changed some physiological properties of *Saccharomyces cerevisiae*. However the test fractions, especially the methanol fractions, were expected to possess greater activity against *S. pombe* since the extract of the *Ganoderma* spp. has well established to possess antitumor and cytotoxic activity (Jong and Birmingham, 1992; Mizuno *et al.*, 1995). One possible explanation for this is that most of the fractions collected are mostly terpenoids. The antitumor activity of *Ganoderma* spp. has been reported to be in the very polar water fraction, where the polysaccharides are present (Mizuno *et al.*, 1995).

Furthermore, even if some polysaccharides were present in the polar methanol fractions, recent investigations have found that the polysaccharides act as a stimulator to the immune system in producing natural killer cells or other lymphocytes, which then kill the tumor cell (Mizuno *et al.*, 1995). The polysaccharide itself, however, does not serve as the potent inhibitory agent against the tumor cell. Thus, the *in vivo* activity of the fractions tested may not equally reflect in the reported *in vitro* experiments.

The same possible explanation may accounts for the antibacterial activity of the fractions tested. Most fractions showed only mild to moderate activity against the test bacteria. Tseng et al (1980) and Hsu et al. (1986) have found that the water extract of *Ganoderma* inhibiting the *in vivo* growth of *Staphylococcus*, *Streptococcus* and *Bacillus pneumonia*. They postulated that the polysaccharides might be the main immune-modulators, increasing the RNA and DNA in the bone marrow in order to produce more of the antibodies in the cells.

5.3.2 Activity of Isolated Compounds

Investigation of antibacterial and antifungal activity of the isolated pure compounds showed that only DEHP (GM 2.2 and GM 3.1.2) from the phthalates family showed moderate activities against both fungus and Gram positive bacteria, *B. subtilis*. This broad-spectrum activity of DEHP agree well with its usage as a minor component in some of the insecticides (Chuah, 1999).

On the other hand, the three sterols, ergosterol, stellasterol and **compound 1** showed only weak activity against one or more of the test microorganisms. Among the three compounds, **compound 1** showed a slightly higher activity against the *Bacillus* spp., with inhibition zone of 8.00 mm as compared to ergosterol and stellasterol (Table 5.2). This may be explained by the present of Br in the structure, which may act as the inhibition agent.

The paper disc diffusion method was chosen as the bioassays test because it can screen large number of samples in a short period of time. More importantly, it is cheap and can be readily carried out in the laboratory. Limitation of this test is that the inhibition zones can sometime be not well defined, thus difficult to interpret and measure quantitatively. In addition, the agar disc diffusion cannot mimic the *in vivo* environment.

Although the screening method used was very simple, the accuracy and precision of the test was based on few critical components. This included the culture medium, the paper discs, inoculum, and other factors such as temperature of incubation, uniformity of agar depth and incubation period (Bridson, 1995).

In short, the paper disc diffusion method can only serve as a preliminary guide for identifying the active fractions in a short period of time. Due to time constraint, minimum inhibition concentrations (MIC) for DEHP and **compound 1** were not made. Following the promising results of bioactive fractions tabulated in Table 5.5, in particular the broad-spectrum activity of fractions GM 3.1-3.5, Fr 5 (MeOH) and Fr 8 (MeOH), further purification should be carried out to characterize the active ingredients.